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# Determination of schizandrin in rat plasma by high-performance liquid chromatography–mass spectrometry and its application in rat pharmacokinetic studies

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**Abstract**

A sensitive liquid chromatography–mass spectrometric (LC/MS) method for the quantification of schizandrin in rat plasma was developed and validated after solid-phase extraction (SPE). Chromatographic separation was achieved on a reversed-phase Shimadzu $C_{18}$ column with the mobile phase of acetonitrile–sodium acetate (10 μmol/L) and step gradient elution resulted in a total run time of about 11.7 min. The analytes were detected using an electrospray positive ionization mass spectrometry in the selected ion monitoring (SIM) mode. A good linear relationship was obtained in the concentration range studied (0.005–2.000 μg/mL) ($r = 0.9999$). Lower limit of quantification (LLOQ) was 5 ng/mL and the lower limit of detection (LLOD) was 2 ng/mL using 100 μL plasma sample. Average recoveries ranged from 75.85 to 88.51% in plasma at the concentrations of 0.005, 0.100 and 1.000 μg/mL. Intra- and inter-day relative standard deviations were 5.95–12.93% and 3.87–14.53%, respectively. This method was successfully applied for the pharmacokinetic studies in rats.


*Keywords:* Schizandrin; Plasma; LC/MS; Solid phase extraction (SPE); Pharmacokinetics

## 1. Introduction

Schisandra berry, fruits of *Schisandra chinensis* (TURCZ.) BAILL. (Schisandraceae), is a famous traditional Chinese medicine, which widely used as a tonic and sedative in China, Korea, Japan and Russia. There are 25 species of the schisandra genus worldwide, while only two of them, the fruits of *S. chinensis* from the northern parts of China (Beiwuweizi) and fruits of *Schisandra sphenanthera* from the southern China (Nanwuweizi), are used in the therapeutic practice. In traditional Chinese and Japanese medicines the drug is used as an antihepatotoxic [1–5], antioxidant and detoxificant [6,7], antidiabetic [8], sedative and tonic agent [9–11].

More than 40 lignan compounds were isolated from schisandra plants [12,13], and schizandrin (Fig. 1) is the major lignans in *S. chinensis* [14]. It has been reported to protect against $CCl_4$-induced hepatic damage [15], and may have some effect on central nervous system (CNS) through activating the cholinergic system or modulate glutamate transporters and reduce extracellular glutamate levels [16–18]. In Chineses Pharmacopeia, schizandrin is defined as the quality control specification for the *S. chinensis* [19] and in many Chinese traditional medicines which contain *S. chinensis*, quantity of schizandrin will also be assayed to control the quality of the compound recipe [20–22].

Since 1980s, much attention has been paid to the pharmacokinetic studies of schizandrin, and many analytical methods were applied in assaying the process of schizandrin in rats or humans, such as thin-layer chromatography (TLC) [23], high-performance liquid chromatography (HPLC) [24–26], nuclear magnetic resonance (NMR) [27], micellar electrokinetic capillary chromatography (MEKC) [28] and gas chromatography coupled with mass spectrometry (GC/MS) [29]. While in recent years, high-performance liquid chromatography coupled with mass spectrometry (LC/MS) are used widly in the analysis of

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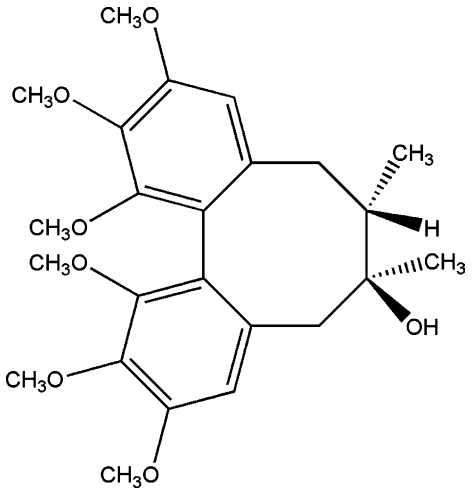


Fig. 1. Chemical structure of schizandrin.

biological samples, which enables us to obtain both of the profits of efficient separating power and obtaining lower limit of detection. This paper introduced a sensitive and specific method using LC/MS for determining schizandrin in rat plasma samples.

## 2. Experimental

### 2.1. Chemicals and reagents

Schizandrin (purity > 98.0%) was kindly provided by Professor Danni Zhu from China Pharmaceutical University. Lovastatin (internal standard) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP). Water was produced by Milli-Q water system (Millipore, Bedford, MA, USA). Acetonitrile (Fisher Company, USA) and methanol (Merck, Germany) were of HPLC grade, sodium acetate is commercially available and was of analytical grade.

### 2.2. Animals

Experimental animals used were Sprague–Dawley rats, weighting 180–220 g of both sex, from the Jiangpu animal breeding center, Nanjing and the studies were approved by the Animal Ethics Committee of China Pharmaceutical University.

### 2.3. Preparation of stock solutions and standards and quality control samples

Stock solution of schizandrin was prepared in methanol at concentration level of 10 mg/mL and lovastatin was prepared in methanol at the concentration level of 1 mg/mL, stored at 4 °C until use. "Individual working solutions" were prepared by diluting stock solutions in 95% methanol for optimization of chromatographic and MS conditions. The working solution for internal standard (2.5 μg/mL) was prepared by diluting the stock solution with methanol. All schizandrin and lovastatin solutions were stored at 4 °C in polypropylene bottles.

Rat plasma calibration standards of schizandrin were prepared by adding 20 μL working solution into 80 μL of drug free rat plasma and well mixed. For schizandrin, concentration points were 0.005, 0.010, 0.050, 0.100, 0.250, 0.500, 1.000 and 2.000 μg/mL. These concentration ranges cover the plasma concentrations expected in our experimental studies. Quality control (QC) samples were prepared in the same way as calibration standards with blank plasma, and QC sample concentrations were 0.005, 0.100 and 1.000 μg/mL. QC samples were stored in polypropylene tubes at −80 °C until analysis.

### 2.4. HPLC/MS analysis

The assay was performed using Shimadzu (Japan) LC/MS 2010A system. Liquid chromatographic separations were achieved using a Shimadzu 3.5 μm $C_{18}$ column (250 mm × 2.00 mm) that was preceded by a guard column ($C_{18}$, 30 mm × 2.00 mm, Phenomenex, Torrance, CA, USA). The column and autosampler tray temperature were kept constant at 40 and 4 °C, respectively. The mobile phase consisted of a mixture of 10 μmol/L sodium acetate in water (A) and acetonitrile (B), and was delivered at a flow-rate of 0.2 mL/min. The gradient program was from 58% B and hold for 3.5 min then to 85% B within 0.1 min at a flow rate of 0.2 mL/min. The sample injection volume was 10 μL.

Samples were ionized by positive-ion electrospray ionization (ESI) probe in the positive ion mode under the following source conditions: gas flow: 4.5 L/min; curve dissolution line (CDL) voltage was fixed as in tuning, CDL temperature: 250 °C; block temperature: 200 °C. Mass spectra were obtained at a dwell time of 0.2 and 1 s for SIM and Scan mode accordingly. Analysis was carried out using selected ion monitoring (SIM) for specific $m/z$ 455.20 for schizandrin $[M + Na]^+$ and 427.20 for lovastatin $[M + Na]^+$. Peak areas for all components were automatically integrated using LC/MS solution Version 2.04 (© 1997–2002 Shimadzu Corp.).

### 2.5. Sample preparation

Venous blood samples (0.2 mL) were withdrawn to the heparinized Vacutainer tubes, and were at once centrifuged at 800 × $g$ for 10 min at 4 °C. A 100 μL volume of plasma was finally obtained, and stored at −20 °C until analysis.

A 100 μL volume of blank plasma, calibration standards, QC samples, and plasma samples were added by 100 μL 50% (v/v) methanol aqueous solution and centrifugated at 10,000 × $g$ for 10 min. All supernatant was loaded and drawn through by gravity on an SPE cartridge ($C_{18}$, HLB 1 cc, Oasis™, Waters, Milford, MA, USA), which was pre-conditioned by passing through 2 mL of methanol followed by 2 mL of water and 20 μL internal standard (lovastatin) before loading, and drawn through by gravity. Then, the solid-phase cartridge was wash with 2 mL of water and 1 mL 30% (v/v) methanol aqueous solution, and finally the SPE cartridge was slowly eluted by 1 mL of 95% (v/v) methanol aqueous solution. The eluent was centrifugated at 10,000 × $g$ for 10 min, and 10 μL of the supernatant fluid was injected into the HPLC/MS system.

### 2.6. Assay validation

#### 2.6.1. Sensitivity and specificity

The lower limit of quantitation (LLOQ) was determined as the minimum concentration that could be accurately and precisely quantified (lowest data point of the standard curve). The lower limit of detection (LLOD on column) was defined as the amount that could be detected with a signal-to-noise ratio of 3. The specificity of the assay for the analytes versus endogenous substances in the matrix was assessed comparing the lowest concentration in the calibration curves with reconstitutions prepared with drug-free plasma from five different rats.

#### 2.6.2. Accuracy and precision

The accuracy and precision (presented as relative standard deviation, R.S.D.) of the assay were determined using QC samples (at 0.005, 0.100 and 1.000 μg/mL). Accuracy (%) was determined from the percentage ratio of measured over spiked QC concentration (mean of measured/spiked × 100%). Intra-day precision was determined by analyzing replicate aliquots of QCs ($n = 5$ per each concentration) on the same day. Inter-day precision was determined by repetitive analysis of QC samples (each concentration) on 5 consecutive days.

#### 2.6.3. Recovery and ionization

To determine the recovery of schizandrin by the SPE method, plasma samples were spiked with schizandrin at concentrations of 0.005, 0.100 and 1.000 μg/mL. The resulting peak–area ratios (analyte:internal standard) were compared with that of the standards prepared in mobile phase to provide the recovery values.

Ion suppression of ionizaition was evaluated by comparing the absolute peak areas of control plasma extracted and then spiked with a known amount of drug, to neat standards injected directly in the same reconstitution solvent.

#### 2.6.4. Stability

To evaluate sample stability after three freeze–thaw cycle and at room temperature, five replicates of QC samples at each of the low, medium and high concentrations were subjected to three freeze–thaw cycles or were stored at room temperature for 4 h before sample processing, respectively. Five replicates of QC samples at each of the low and high concentrations were processed and stored under autosampler conditions for 24 h. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of freshly prepared QC samples.

#### 2.6.5. Application of the assay

The developed LC/MS assay method was used in the pharmacokinetic study after intravenous (i.v.) (1 mg/kg) and intragastic (i.g.) (10 mg/kg) administration of schizandrin to rats. Animals were fasted for 12 h before dosing and 4 h afterwards, with free access to water. For intravenous bolus, schizandrin powder was dissolved in isotonic saline containing 10% ethanol and dosing solution was delivered using a 1 mL syringe into a rat's tail vein. For oral route, dosing solutions were prepared by dissolving schizandrin powder in isotonic saline containing 20% ethanol and mixed well. The preparations were made immediately before drug administration. Under ether anesthesia, a cannula was inserted into the rat's jugular vein. Blood samples (200 μL) were collected immediately before and at 2, 5, 8, 10, 20 and 45 min, 1, 1.5, 2, 3 and 4 h for i.v. group and 2, 5, 8, 15, 30 and 45 min, 1, 1.5, 2, 4 and 6 h for i.g. group after schizandrin administration. After each blood sampling, the cannula was flushed with physiologic saline containing heparin. The blood sample was transferred into a heparinized eppendoff tube and mixed gently, and then centrifuged (1000 × $g$, 5 min) to obtain 100 μL plasma, which was kept at −20 °C until analysis.

## 3. Results and discussion

### 3.1. Liquid chromatography–mass spectrometry

Regarding analytical methods of schizandrin for basic and clinical studies, thin-layer chromatography (TLC) [27], high-performance liquid chromatography (HPLC) [28] and gas chromatography coupled with mass spectrometry (GC/MS) [23] has been described. However, the lower limits of detection (LLOQ) for TLC and HPLC methods were higher than 0.005 μg/mL, and use more plasma. The LLOQ of GC/MS was 0.002 μg/mL, and the eluation of plasma sample after SPE extraction needed to be evaporated to dryness. If necessary, this method could also introduced evaporation, when the LLOQ would reach to 0.0005 μg/mL. Therefore, it is considered that the LC/MS method for schizandrin we developed here will facilitate obtaining quality data for basic pharmacokinetic and pharmaceutical studies.

### 3.2. Mass spectra analysis

The full scan mass spectra of schizandrin and lovastatin after direct injection in mobile phase are presented in Fig. 2. Protonated molecules $[M + H]^+$ or $[M - H]^-$ of schizandrin were

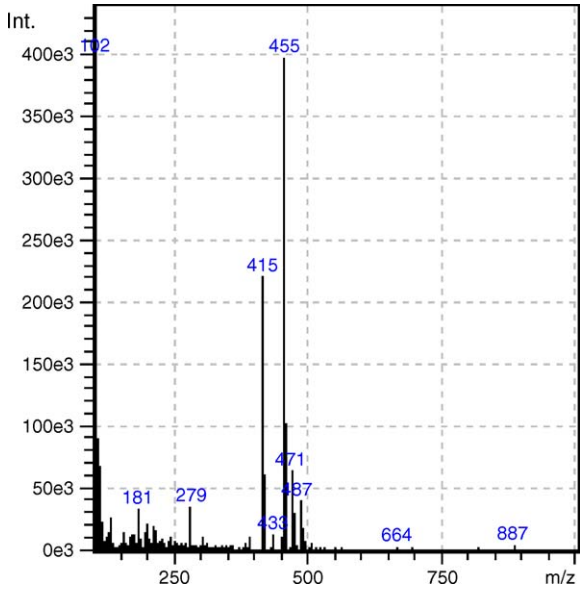


Fig. 2. Full scan mass spectra of schizandrin (1 μg/mL).

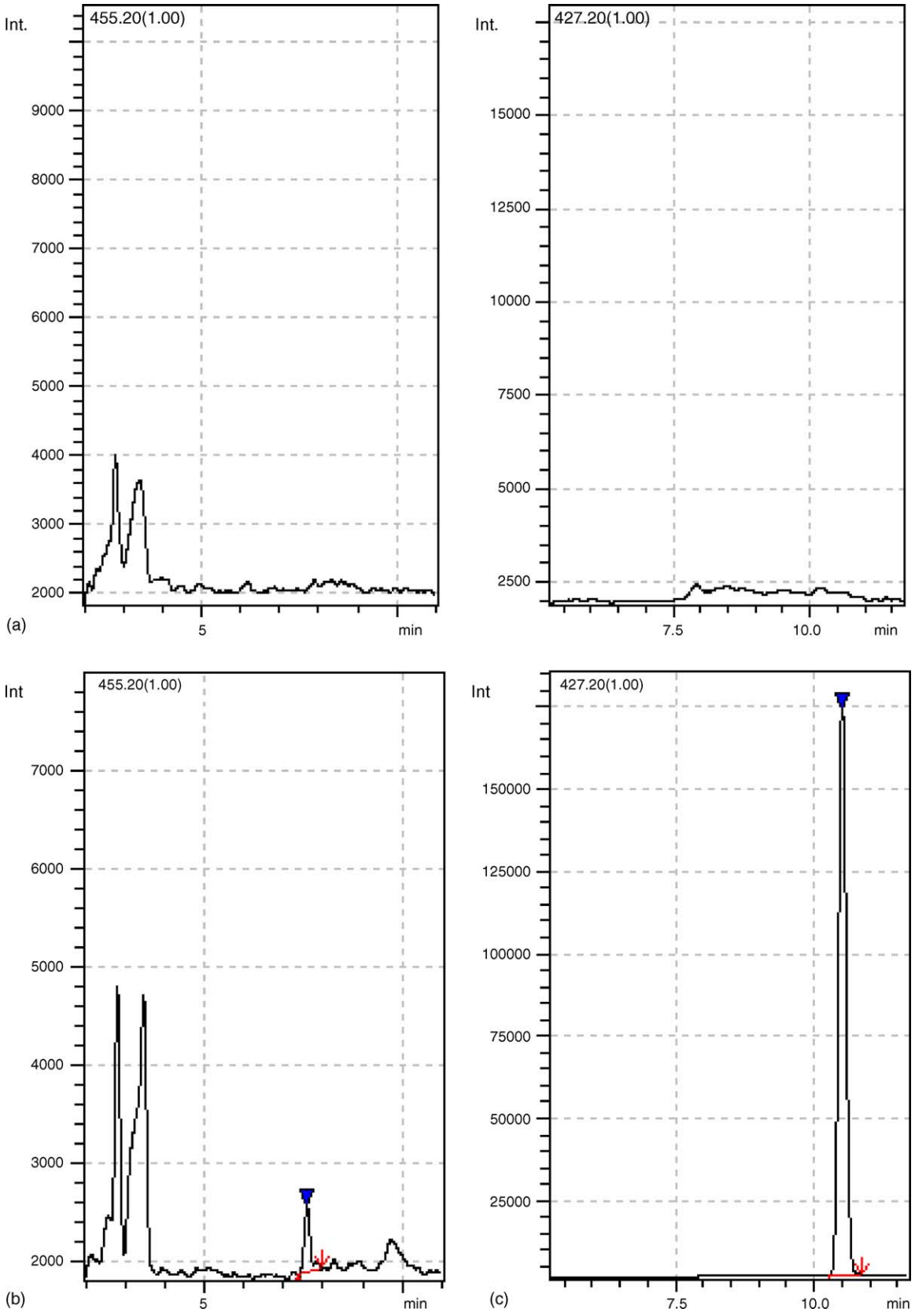


Fig. 3. Representative chromatograms obtained following extraction of (a) blank plasma, (1) schizandrin, (2) lovastatin; (b) blank plasma spiked with schizandrin (5 ng/mL, limit of quantification level); (c) blank plasma spiked with lovastatin (I.S., 2.5 μg/mL); (d) a rat plasma sample 30 min after oral administration of schizandrin (10 mg/kg); (e) a rat plasma sample 30 min after intraveanous administration of schizandrin (1 mg/kg).

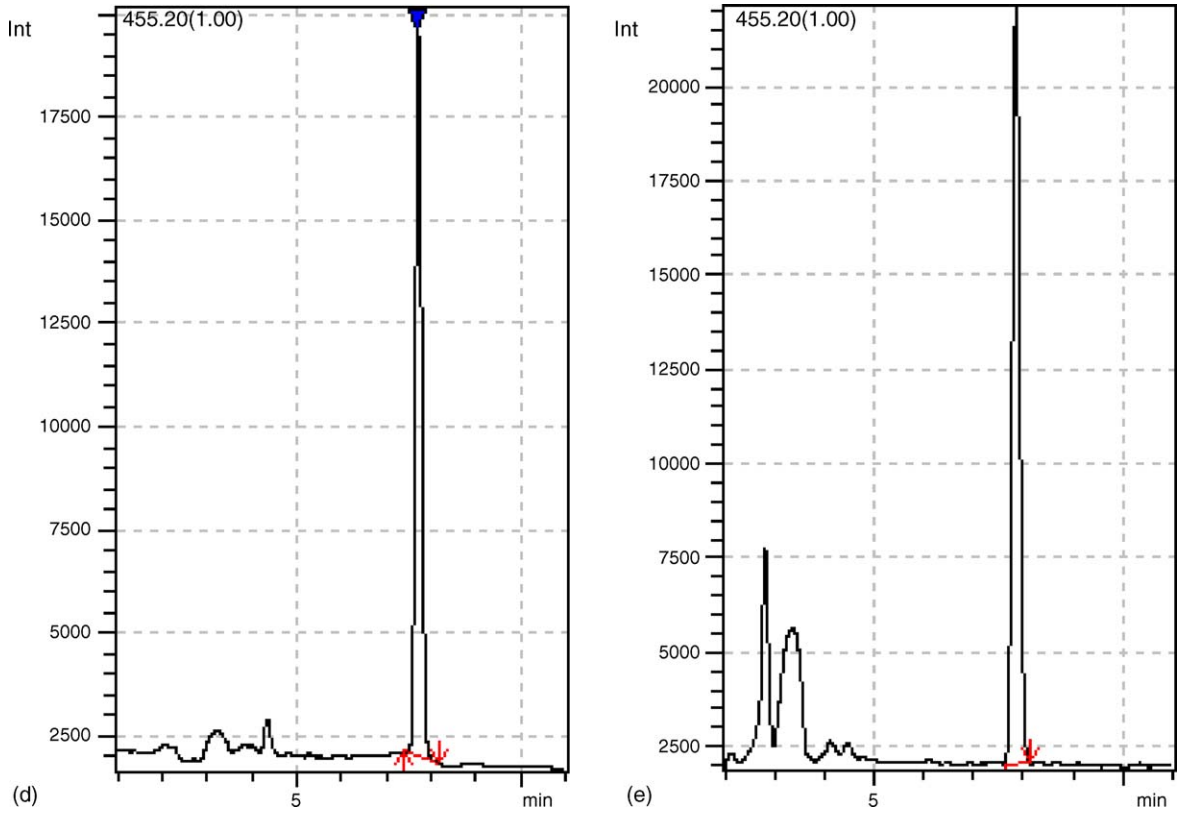


Fig. 3. (*Continued*).

not detected. The predominant protonated molecules found for schizandrin were $[M + Na]^+$ *m*/*z* 455.2 and $[M + H–OH]^+$ *m*/*z* 415.2. The mass spectrometric parameters were optimized to obtain the higher signal for the selected ion 455.2, which also showed less internal interference. The protonated molecule was $[M + Na]^+$ detected for lovastatin at *m*/*z* 427.2.

### 3.3. HPLC gradient programme

While using the gradient programme, observed retention times were about 7.8 and 10.5 min for schizandrin and lovastatin, respectively. No additional peak due to endogenous substances that could have interfered with the detection of the compounds of interest was observed. Representative chromatograms for schizandrin and lovastatin in actual plasma sample are presented in Fig. 3.

### 3.4. Method development for solid-phase extraction

Several extraction procedures were tested including solid-phase extraction (SPE) using HLB cartridge and the liquid–liquid extraction method by ether. It was estimated that SPE shows strong ability to remove excessive interferences and efficiently extract the drug of interest from the plasma sample. Therefore, it was adopted for extracting schizandrin from rat plasma. The purpose of using 1 mL of 30% (v/v) methanol aqueous solution before the finally elution was to wash out the more excessive interferences and obtained the better recovery comparing with other concentrations (40 and 60%). A better recovery was also given when using the 1 mL of 95% (v/v) methanol aqueous solution for eluting the samples than other concentrations (70, 80 and 95%) from the solid-phase column.

### 3.5. Method validation

#### 3.5.1. Linearity

The linear regression analysis of schizandrin was constructed by plotting the peak–area ratio of schizandrin to the internal standard (*y*) versus analyte concentration (μg/mL) in spiked plasma samples (*x*). The calibration curves were constructed in the range of 0.005–2.000 μg/mL. The average regression equation of these curves and their correlation coefficients (*r*) were calculated as follows: $y = 0.4025x + 0.0005$ ($r = 0.9999$, $n = 5$), weighting coefficient: $1/x$; it showed good linear relationships between the peak areas and the concentrations.

#### 3.5.2. Precision

The intra-day precision (presented as relative standard deviation, R.S.D.) is shown in Table 1. The precision for concentrations of 0.005, 0.100 and 1.000 μg/mL schizandrin were 12.93, 9.21 and 5.95%, respectively, and the accuracy, defined as (measured concentration/spiked concentration) × 100%, reached from 87.36 to 103.55% throughout the three concentrations examined.

The inter-day precision was studied over 5 days, and the results were also given in Table 1. The precision ranged from 3.87 to 14.53%, and the accuracy, reached from 94.79 to 103.26% throughout the three concentrations examined. The

Table 1
Precision and accuracy of schizandrin in rat plasma

| Spiked concentration (μg/mL) | Intra-day precision ($n = 5$) | | | Inter-day precision ($n = 5$) | | |
|---|---|---|---|---|---|---|
| | 0.005 | 0.100 | 1.000 | 0.005 | 0.100 | 1.000 |
| Measured concentration (μg/mL) | 0.004 ± 0.001 | 0.104 ± 0.010 | 1.026 ± 0.061 | 0.005 ± 0.001 | 0.106 ± 0.008 | 0.994 ± 0.039 |
| Accuracy (%) | 87.36 ± 10.61 | 103.55 ± 9.53 | 102.64 ± 6.11 | 94.79 ± 13.78 | 106.33 ± 7.90 | 99.44 ± 3.85 |
| R.S.D. (%) | 12.93 | 9.21 | 5.95 | 14.53 | 7.43 | 3.87 |

Table 2
Absolute recoveries of schizandrin and lovastatin (internal standard) from spiked rat plasma

| Concentration (μg/mL) | Recovery (%, mean ± S.D., $n = 5$) | |
|---|---|---|
| | Schizandrin | Lovastatin |
| 0.005 | 81.07 ± 9.35 | – |
| 0.100 | 88.51 ± 3.03 | – |
| 1.000 | 75.85 ± 3.57 | – |
| 2.500 | – | 78.76 ± 4.94 |

lower limit of quantification (LLOQ) was 5 ng/mL for schizandrin.

The absolute recoveries of schizandrin added to rat plasma were from 75.85 to 88.51%, and the absolute recovery of lovastatin added to rat plasma was 78.76 ± 4.94%, which were given in Table 2.

### 3.5.3. Ionization

It was shown that SPE improving the sample clean-up to remove internal substances in plasma and thereby decreasing the amount of matrix injected onto the column, thus the ion suppression effect was minimized. The results showed that there was no significant difference in the signals of analytes extracted from rat plasma and from the mobile phase, indicating that there were no matrix effects.

### 3.5.4. Stability

Stability of schizandrin during sample handling (freeze–thaw and short-term temperature) and the stability of processed samples were evaluated (Table 3). Schizandrin was stable for at least 4 h at room temperature in plasma samples, for 24 h in autosampler conditions and in plasma samples following three freeze–thaw cycles.

Table 3
Stability of samples ($n = 5$)

| Measured concentration (μg/mL) | Spiked concentration (μg/mL) | | |
|---|---|---|---|
| | 0.005 | 0.100 | 1.000 |
| Freeze and thaw stability | | | |
| Mean | 0.004 ± 0.001 | 0.103 ± 0.010 | 0.987 ± 0.031 |
| R.S.D. (%) | 13.40 | 9.61 | 3.17 |
| Short-term temperature stability (4 h at room temperature) | | | |
| Mean | 0.004 ± 0.000 | 0.100 ± 0.005 | 0.997 ± 0.021 |
| R.S.D. (%) | 11.39 | 5.44 | 2.14 |
| Post-preparative stability (24 h at room temperature) | | | |
| Mean | 0.004 ± 0.001 | 0.102 ± 0.009 | 1.008 ± 0.064 |
| R.S.D. (%) | 12.56 | 8.45 | 6.38 |

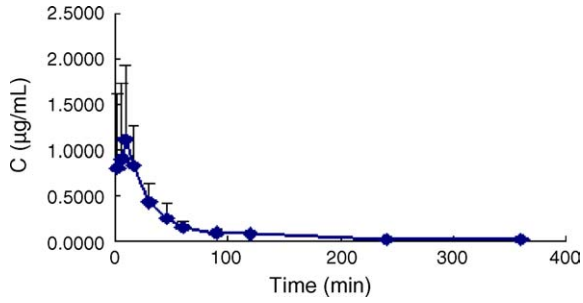


Fig. 4. Mean plasma schizandrin concentration vs. time profiles in six rats after i.g. administration (10 mg/kg).

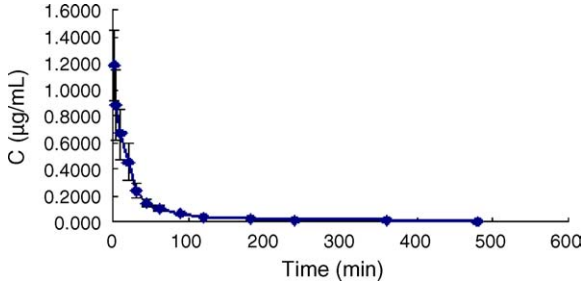


Fig. 5. Mean plasma schizandrin concentration vs. time profiles in six rats after i.v. administration (1 mg/kg).

### 3.5.5. Pharmacokinetic study of schizandrin in rats

The assay was used to obtain pharmacokinetic data for schizandrin in rat plasma after i.v. administration (1 mg/kg) and i.g. administration (10 mg/kg). Figs. 4 and 5 show application of the LC/MS method developed here to in vivo pharmacokinetic studies in rats. The area under the plasma concentration (AUCs curve) of schizandrin after i.v. and p.o. administrations were 0.478 ± 0.110 mg/L h and 0.717 ± 0.397 mg/L h, respectively. The absolute bioavailability ($F$, %) of schizandrin, was found to be 14.8%.

## 4. Conclusion

A sensitive and reliable LC/MS method for the analysis of schizandrin in rat plasma has been successfully developed and validated. To extract schizandrin from the plasma, SPE extraction with methanol aqueous solution was used. This method demonstrated a relatively short analysis time and the acceptable sensitivity, precision, accuracy, selectivity, recovery and

stability. The method was successfully applied to a pharmacokinetic study of schizandrin in rats. And to our knowledge, it is the first report of LC/MS method on the determination of schizandrin concentration in vivo.

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